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14. ABSTRACT

The goal of this study is to test whether ER– breast cancers arise from ER– or ER+ mammary cells. We specifically hypothesize that ER is absent in the originating cell of ER-negative breast cancer. Although until now it has been technically difficult to test it, we have developed a unique mouse model based on the RCAS-TVA technology that allows us to trace the ER status of the cancer-originating cell. It is now possible to test this hypothesis in experimental mice. In this funding period, we have generated mouse lines and viruses for the experiments proposed, and have found that the cell origin of breast cancer affects the ER status of breast cancer. In addition, we have found that somatic activation of growth factor signaling induces estrogen-independent tumor initiation.

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Breast cancer, initiation, estrogen receptor, mouse model, ErbB2

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BC060332: ER/PR status of the originating cell of ER-negative breast cancer

Yi Li, Ph.D.

INTRODUCTION

The goal of this study is to test whether estrogen receptor-negative (ER-) breast cancers arise from ER- or ER+ mammary cells. We specifically hypothesize that ER is absent in the originating cell of ER-negative breast cancer. Although until now it has been technically difficult to test it, we have developed a method that allows us to trace the ER status of the cancer-originating cell. It is now possible to test this hypothesis in experimental mice.

BODY

Task 1. Dertermine whether ER- mammary tumors arise from a PR- normal mammary precursor cell

Strategy. We will infect mammary glands in the MMTV-tva/PR$^{Cre/+}$ female mice with RCAS viruses that express both an oncogene and a GFP reporter that is flanked by the loxP recombination sites and thus can be deleted by the Cre expressed from the PR promoter. Expression of PR in any infected cell will delete proviral GFP and cause this cell and all of its progeny to stop expressing GFP. Thus, if the cell destined to become PR– mammary cancer does not express PR any time prior to full transformation, tumor cells in the resulting cancer will retain GFP. Consequently, GFP expression in these PR– tumors will demonstrate that the tumors arise from a PR– cell. Conversely, lack of GFP in the resulting tumors will reveal that PR is expressed some time in the transition from a normal epithelial cell to malignancy.

Progress. We have generated strong evidence that in MMMTV-tva mice, RCAS can infect both ER+ and ER- cells. Using co-immunofluorescent staining 4 days following intraductal injection of RCAS-GFP into adult tva transgenic mice, about 40% GFP+ cells were found to express ER, similar to the frequency in non-infected cells (Figure 1).

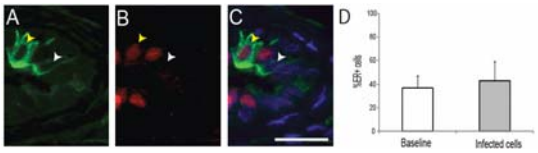


Figure 1. GFP is detected in ER+ and ER- mammary cells 4 days following infection of MMTV-tva adult mice with RCAS-GFP virus. GFP (green), ER (red).

We have made RCAS-PyMT-flox-IRES-GFP (RCAS-PfiG). However, infection of MMTV-tva mice with this virus resulted in tumors that did not lead to proper GFP expression tumor cells. We have also made RCAS-Neu-flox-IRES-GFP (RCAS-NfiG). While infection of MMTV-tva mice with this virus resulted in early lesions, only one of the six mice infected with this virus developed tumors after one year, and the tumor did not have detectable Neu though GFP could be detected. We suspect that these viruses are not stable in vivo due to the large insert size (2.8 kb) since the expression of genes in RCAS is optimal when it is <2.5kb.

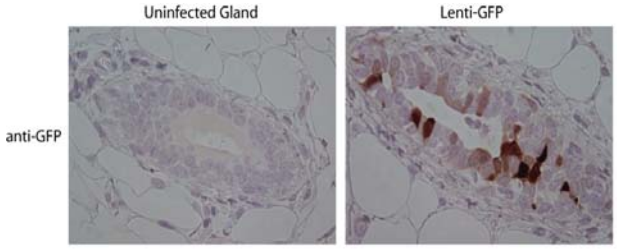


Figure 2) FVB mice were infected with lent-GFP (contralateral glands as negative control). 4 days later, mice were sacrificed. IHC for GFP above.

Therefore, we have opted to use a lentiviral vector to introduce the NfiG insert due to its ability to accommodate a larger insert size. Although we have previously reported RCAS env pseudotyped HIV vector for mammary gland gene delivery[1], we have not been able to achieve a sufficient titer for this purpose. Therefore, we decided to use VSV-G pseudotyped HIV vectors. These vectors can grow to high titers, but they will lose the cell type specificity associated with the use of the RCAS/TVA technology. Since the delivery method restricts the infection to the ductal epithelium, it should be fine for our specific purpose here. In collaboration with Dr. Lei Li at MD Anderson Cancer Center, we inject a third generation lenti-GFP

intraductally into non-transgenic FVB mice. Four days later, the mice were sacrificed and IHC for GFP was performed (Figure 2). Indeed we observed a high number of cells contained GFP, showing that lentiviral infection results in somatic infection in mice.

We have started to clone the NfiG insert into this vector, and we will confirm the expression of both Neu and GFP in vitro. Then, we will use them to infect 20 PR^{Cre} mice, which we have already imported, to test whether the resulting tumors will express GFP or not, so as to conclude whether these tumors originated in PR- cells or not.

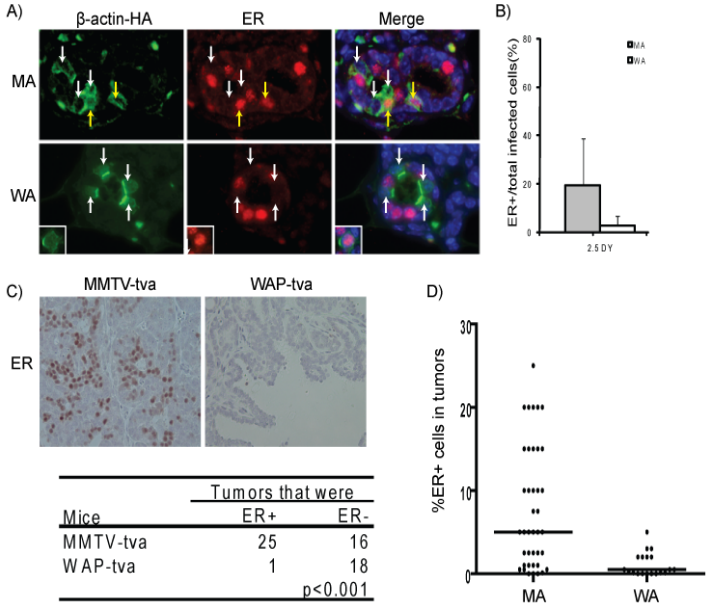
Besides using Neu as the inducer of tumors, we proposed to test select other oncogenes. We have found that c-Myc delivered by RCAS failed to induce mammary tumors in MMTV-tva mice. After we have shown that the lentivirus vector works well in the above tests, we will try use it to express c-Myc-floxed GFP or EGFR-floxed GFP

Task 2. Determine w hether ER- mamma ry tumors ar ise from a n ER- precursor cell in the normal mammary epithelium

We proposed to create ER-Cre BAC transgenic mice, breed with MMTV-tva mice, and use the strategy in Task 1 to test whether ER– mammary tumors arise from an ER– precursor cell in the normal mammary epithelium. The Cre protein is difficult to detect in vivo. Therefore, we first cloned a reporter gene (tva encoding a protein product that can be easily detected by IHC) into the best ER BAC we could find, and made two BAC transgenic founders. However neither founder line expressed tva at detectable levels. Therefore, the best ER BAC clone available at this time cannot express an exogenous gene at a detectable level. Thus, the knock-in approach appears to be a necessary alternative.

However, while we were in the process of starting to make TVA knock-in, we found the following surprise result that provides an alternative approach to our goal in this Aim. We have been focusing on this alternative approach in the past one year:

We have also created a transgenic line expressing tva from the whey acidic promoter (WAP), in addition to the MMTV-tva line we reported previously[2]. This allows us to somatically introduce oncogenes into distinct mammary cell populations between the two lines. We have generated tumors by infection with RCAS-PyMT in both lines. Tumors arising in MMTV-tva mice were predominately ER+ (61%) (Fig 3C&D); however, 95% of tumors arising in WAP-tva mice were ER- (Fig 3C). Interestingly, when we compared the cells infected with RCAS-β-actin (HA-tagged) between the two lines, we found that while infected cells (defined by positive HA staining) in MA mice consisted of both ER+ (20%) and ER- (80%) cells 2.5 days after infection, 97% of infected cells in WA mice were ER- (Fig



Mice	Tumors that were ER+	ER-
MMTV-tva	25	16
WAP-tva	1	18
		p<0.001

Figure 3 A) Adult MMTV-tva (MA) and WAP-tva (WA) mice were infected with RCAS-β-actin (HA-tagged). 2.5 days later mice were sacrificed, and co-IF for HA and ER was performed. Cells with co-localization of HA and ER are indicated by yellow arrows. B) 20% of HA+ cells were ER+ in MA mice (n=8). Only 3% of HA+ cells were ER+ in WA mice (n=9)(p=0.04). C) 61% of tumors induced by RCAS-PyMT in MA mice express ER in at least 5% of cells. 5% of tumors in WA mice were ER+ (p<0.001). D) Percentage of ER+ cells per tumor between MA and WA mice. Median shown by horizontal bar.

3A&B). These results strongly suggest that the ER- tumors induced by RCAS-PyMT in WAP-tva mice originated in ER- cells. We are currently investigating that the molecular features of these infected ER- cells in these WAP-tva mice, and plan to submit a manuscript soon.

After we are done with the above alternative, we will come back to making and testing Cre knock-in into the ER locus.

Task 3. D etermine molecular differences in response to oncogene activa tion betw een ER- and ER+ mammary cells in the normal mammary gland

We have established proper cell isolation method and FACS protocol for primary mammary cells. We have isolated infected cells in WAP-tva mice injected by RCAS-GFP. We will compare these cells with infected cells isolated from MMTV-tva mice injected by RCAS-GFP by several methods including cell differentiation markers and mammosphere. This comparison will help us learn the unique features of cells giving rise to ER- tumors.

After the lentivirus expressing both Neu and GFP is made in Task 2, we will use it to isolate Neu-activated cells and compare with cells infected by lentivirus making only GFP study their unique properties using similar methods.

<u>Other findings from work not initially proposed:</u>
Somatic activation of growth factor signaling induces estrogen-independent tumor initiation. (near ready for submission)

Abstract: Estrogen signaling is required for proliferation of all normal mammary epithelial cells; yet prophylactic blockage of estrogen signaling fails to prevent breast carcinogenesis in 30% of the patients that otherwise would develop breast cancer. Studies in germline mouse models have shown that loss of estrogen signaling blocks carcinogenesis in mammary cells that have lost p53 or gained an oncogene such as ErbB2. However, we find that in a somatic mouse model that more closely mimics the evolution of sporadic human breast cancers, mammary epithelial cells that have gained PyMT or ErbB2 evolve into tumors independent of estrogen or other ovarian functions. Our data suggest that prophylactic estrogenic therapy cannot prevent carcinogenesis in mammary epithelial cells that gained certain genetic alterations, especially those encoding components of the growth factors signaling pathways.

A more detailed description of the critical data is as follows:

PyMT gave rise to predominately ER+ tumors (61%), while ErbB2 gave rise to predominately ER-

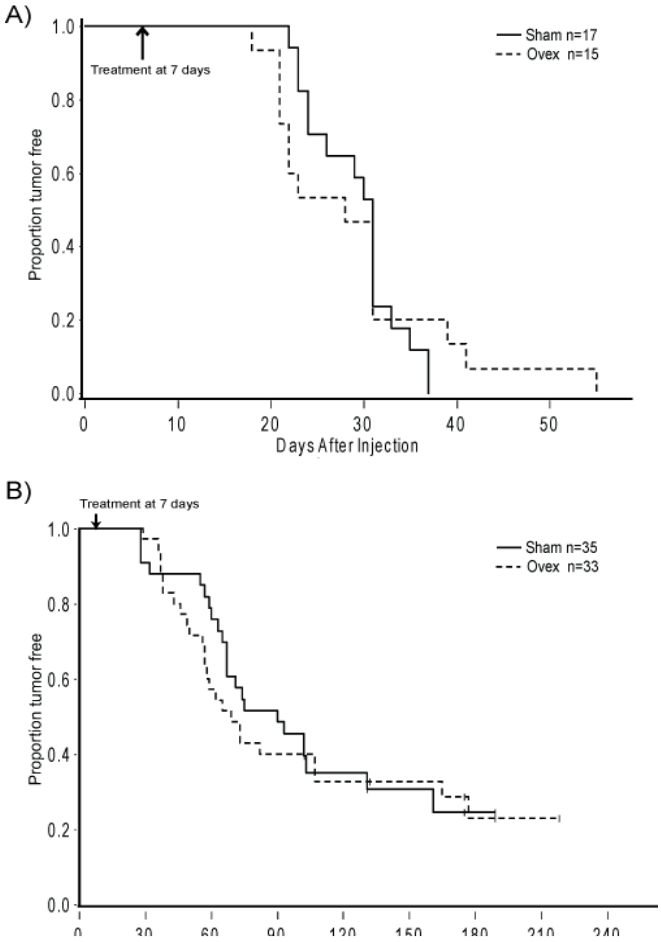


Figure 4 . Kaplan-Meier plots demonstrating estrogen-independent initiation of tumors induced by activation of growth factor signaling pathways in mice. Adult mice were randomized and ovariectomized 1 week post-infection. Tumor initiation was monitored for first appearance of tumors. A) Tumors induced by RCAS-PyMT were unable to be prevented by ovariectomy (p=0.55). B) Tumors induced by RCAS-ErbB2 were unable to be prevented by ovariectomy (p=0.44)

tumors (79%). Estrogen deprivation by ovariectomy was unable to prevent or delay tumors induced by either oncogene (Figure 4), suggesting that somatic activation of growth factor signaling pathways induces estrogen-independent tumor initiation. We found that ErbB2 causes normally quiescent ER+ cells to proliferate (6.1±2.9% vs. 0.8±1.3% in non-infected ER+ cells; p=0.002)(Figure 5a-b). After the gain of ErbB2, the majority of ER+ cells, in addition to ER- cells, which comprised the large majority of proliferating cells in early lesions (91±8%), no longer depend on estrogen for proliferation (Figure 5c).

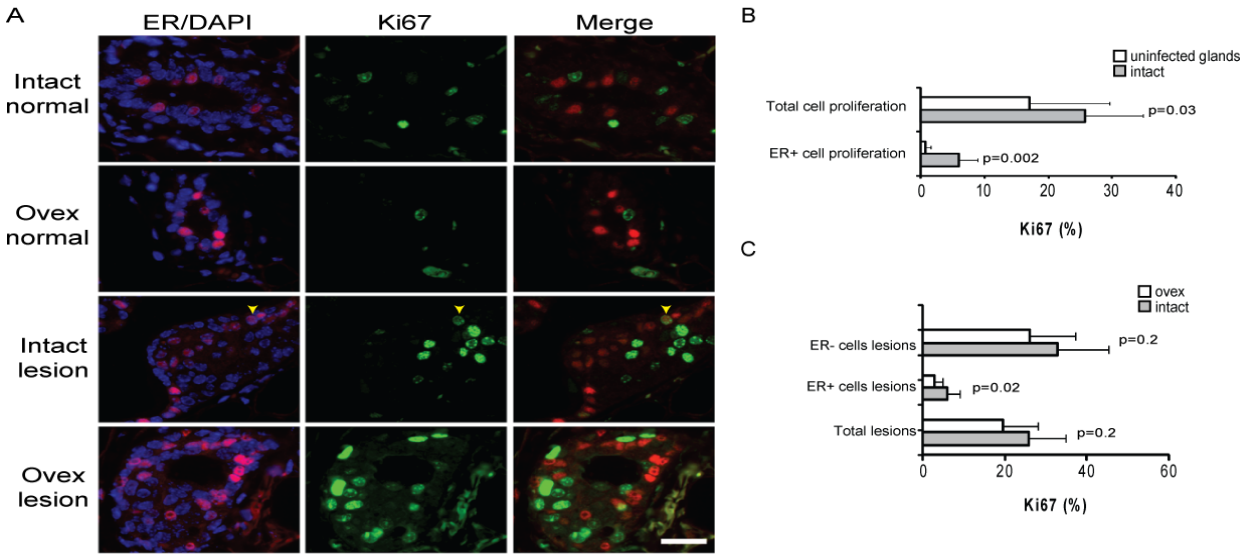


Figure 5. 12-16 week old-mice (n=18) were infected with RCAS-ErbB2 (3 glands/mouse). 1 week later, mice were randomized and ovariectomy or sham surgery was performed. Mice were sacrificed 2 weeks after ovariectomy. Non-infected glands were used as negative control. (A) Co-immunofluorescence was performed for ER and Ki67. Co-localized cells indicated by yellow arrow. B) Normally quiescent ER^+ cells (0.8±1.3%, uninfected glands) are induced to proliferate by ErbB2 (6.1±2.9%) (p=0.0004). C) Ovariectomy has no effect on the proliferation of the large majority of $ErbB2^+$ cells. However, the proliferation of a subset of ER^+ cells in early lesions is affected by estrogen deprivation (6.1±2.9% in intact mice compared to 2.85±1.96% in ovariectomized mice, p=0.02). Scale bar=20μm.

KEY RESEARCH ACCOMPLISHMENTS:

- Produced strong evidence that both ER+ and ER- cells can be infected by RCAS viruses.
- Provide evidence that ER- cells are the cell of tumor origin of ER- tumors induced by RCAS-PyMT
- Demonstrated that somatic activation of growth factor signaling induces estrogen-independent tumor initiation

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

- The 2008 DOD Era of Hope meeting poster presentation. Toneff et al: Somatic activation of growth factor signaling induces estrogen-independent tumor initiation
- Gordan Conference on Cancer Models and Mechanisms. Bryant College. 7/27-8/1/2009. Toneff et al: Somatic activation of growth factor signaling induces estrogen-independent tumor initiation.
- The 31st Department of Molecular and Cellular Biology Graduate Student Symposium, Baylor College of Medicine. 4/29-30, 2009. Toneff et al: The ER-status of the cell of breast cancer origin affects tumor ER-status.

CONCLUSIONS:

We have created valuable reagents for the experiments proposed, and have found that the cell origin of breast cancer affects the ER status of breast cancer. In addition, we have found that somatic activation of growth factor signaling induces estrogen-independent tumor initiation.

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APPENDICES

none